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자외선B가 조사된 사람 각질 세포주에 있어서 interleukin-22의 효과

The effect of interleukin (IL)-22 on human keratinocyte cell line irradiated by UVB

2014년 2월

서울대학교 대학원

의학과 해부학 전공

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The effect of interleukin (IL)-22 on human keratinocyte cell line irradiated by UVB

by

Yejin kim

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Anatomy) at Seoul National University College of Medicine

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ABSTRACT

The effect of interleukin (IL)-22 on human keratinocyte cell line irradiated by UVB

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Interleukin (IL)-22, a member of IL-10 family, is a potent mediator of inflammatory responses. It is produced by activated $CD4^+$ T cells and natural killer (NK) cells, but IL-22Rα expression is restricted to nonhematopoietic cells in the skin, pancreas, intestine, liver, lung and kidney. It has recently been reported that IL-22 plays a critical role in the maintenance of epidermal homeostasis by controlling cell cycle of keratinocytes. In addition, it is already known that ultraviolet B (UVB) radiation induces skin inflammation. However, there are no reports regarding the role of UVB on the production of IL-22 and its receptor expression. Therefore, I investigated the role of IL-22 on the proliferation of UVB-irradiated human keratinocyte cell line, HaCaT and the induction of skin inflammation in terms of IL-22Rα expression on HaCaT. The expression of *IL-22Rα* mRNA and its protein in

HaCaT was increased by UVB (100 J/m^2) irradiation. Interestingly, the translocation of IL-22Rα from cytosol to membrane was increased by UVB irradiation. It is generally known that UVB suppresses the proliferation of HaCaT, but the suppressed proliferation of UVB-irradiated HaCaT was recovered by the treatment of recombinant IL-22 and culture supernatant of activated PBMCs. Finally, the production of pro-inflammatory cytokines, such as IL-1α, IL-6 and IL-18, was increased from UVB-irradiated HaCaT by the treatment of rIL-22. Taken together, IL-22 increases skin inflammation and the proliferation of HaCaT through the interaction with up-regulated IL-22Rα on HaCaT by UVB irradiation.

Keywords: inflammation, proliferation, HaCaT, IL-22, UVB

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LIST OF ABBREVIATIONS

Con A: concanavalin A

ELISA: enzyme-linked immunosorbent assay

HRP: horse radish peroxidase

IL: interleukin

NK cell: natural killer cell

pAkt: phosphorylated Akt

PBMCs: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PBST: PBS containing 0.1% Tween 20

PFA: paraformaldehyde

PI3K: phosphoinositide 3-kinase

rIL-22: recombinant interleukin-22

RT-PCR: reverse transcription-polymerase chain reaction

Th2: type 2 helper T cell

UVB: ultraviolet B

INTRODUCTION

There are three types of ultraviolet (UV) rays; UVA (wavelength, 320-400 nm), UVB (280-320 nm) and UVC (180-280 nm) (1). UVA has longer wavelength but lower energy potential than UVB. For this reason, UVA causes skin aging and chronic skin damages after penetrating into the dermis (2, 3). UVB is closely related with the development of skin cancer caused by DNA damage, such as the formation of thymidine (T-T) dimers, 6, 4-pyrimidine-pyrimidine photoproducts and single strand breaks (4-6). It is also involved in the induction of acute inflammatory responses in the skin through the production of inflammatory mediators from keratinocyte and functional alteration of immune cells in the skin (7, 8). In case of UVC, it does not reach the surface of the Earth because of its absorption by ozone layer in the stratosphere (9, 10). It is known that acute skin inflammation by UVB irradiation is mediated by the secretion of inflammatory cytokines such as IL-1,-6,-8,-10 and tumor necrosis factor (TNF-α) (11). There have been several reports regarding the involvement of the activation of p38 MAPK, ERK and JNK in this process (12, 13). We have recently reported the regulatory mechanisms on the production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and IL-18 through the MAPK signaling cascades in human

keratinocyte cell line, HaCaT (14). Since reactive oxygen species (ROS) play an important role in this process, it is believed that the prevention of ROS production by UVB irradiation is an effective treatment of skin diseases, even in cancer (15). In fact, there are numerous reports about the effective prevention and therapy of UVB-induced skin inflammation and cancer by using anti-oxidant molecules, such as vitamin C, resveratrol, and polyphenol. (16-18).

Interleukin (IL)-22 was discovered as a member of IL-10 cytokine family (19). It is mainly produced by activated $CD4^+$ T cells and natural killer (NK) cells (20, 21). IL-22 receptor (IL-22R) is composed of IL-22Rα and IL-10Rβ. IL-10Rβ is ubiquitously expressed whereas IL-22Rα expression is restricted to nonhematopoietic cells, such as the cells in the skin, pancreas, intestine, liver, lung and kidney (22). IL-22 has a potent inflammatory and proliferative effect in different cell lines (23-25). It has been reported that IL-22 also plays an important role in inflammatory processes and wound healing processes in the skin (24, 26, 27). In addition, IL-22 induces the proliferation of normal human epidermal keratinocytes obtained from healthy individuals and fibroblast like synoviocytes isolated from psoriatic arthritis, rheumatoid arthritis and osteoarthritis patients (23, 28, 29). Even though IL-22 responsiveness is dependent on PI3K/Akt/mTOR signaling pathway, IL-22 also activates the Janus kinase 1 (JAK1), signal transducer and activator of

transcription protein 3 (STAT3), p38 mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal regulated kinase 1/2 (ERK 1/2) to execute its function (28, 30). In case of IL-22R, its expression is enhanced in pathological environment. For example, IL-22R expression is increased in keratinocyte by IFN-α and is considered one of the innate immunity against viral infection (31, 32). However, when IL-22R is overexpressed, IL-22 inhibits terminal differentiation of keratinocyte and causes thickening of the epidermis (31). Furthermore, the expression of *IL-22Rα* mRNA is significantly increased in psoriatic epidermis, whereas there is no change in the expression of *IL-10Rβ* mRNA (33, 34). It suggests that the increased expression of IL-22Rα is closely related with the acute and chronic skin diseases. It is known that IL-22Rα expression is regulated by the activation of PI3K/Akt pathway (35).

It is clear that IL-22 production from $CD4^+$ T cells is stimulated by IL-6 and IL-23 during bacterial infection (36, 37). However, it is still not known what kinds of molecules are involved in the regulation of IL-22Rα expression, especially on the skin. Moreover, the effect of interaction between IL-22 and its receptor on the process of UVB-induced skin inflammation is yet to be discovered. I here, therefore, investigate whether UVB facilitates inflammatory responses in the skin through the increase of IL-22Rα

expression and the responsiveness to IL-22 in HaCaT.

MATERIALS AND METHODS

Cell culture The human keratinocyte cell line, HaCaT is grown in RPMI1640 (WElGENE, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY, USA) and antibiotics (100 U/ml of penicillin and 100 μg/ml streptomycin; Life Technologies) at 37 ^{o}C in a humidified incubator containing 5% CO_2. This cell line was used for the experiments while in the log phase of growth. HaCaT was kindly provided by Dr. Kyung Chan Park (Department of Dermatology, Seoul National University College of Medicine) and originally developed by Fusenig.NE (38).

UVB irradiation Cells (7.5×10^5) were grown in culture dish up to 70-80% confluence and washed with phosphate buffered saline (PBS) prior to UVB irradiation using the XL-1000 UV Crosslinker (Spectronics Corporation, Westbury, NY, USA). UV doses determined using an UV light meter; YK-34UV (Lutron, Coopersburg, PA, USA). After exposure to UVB, cells were replenished with media containing 10% heat-inactivated fetal bovine serum and harvested at the time point indicated.

Measurement of cell viability and % of cell growth rate by UVB Cells

(7.5×10^5) were exposed to 100, 150, and 200 J/m^2 of UVB, and incubated with media containing 10% heat-inactivated fetal bovine serum for 24 hrs. And then cell viability and % of cell growth were measured by trypan blue dye exclusion assay; cell viability (%) = (the numbers of live cells/ the numbers of total cells) × 100. Triplicate dishes are averaged and % of cell growth was calculated as follows: % of cell growth = (the numbers of live cells at 24 hr after UVB irradiation/ the numbers of live cells at 24 hr without UVB irradiation) × 100.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) To study the expression of *IL-22Rα* and *IL-10Rβ* in UVB-irradiated HaCaT, RT-PCR was performed. Briefly, cells (1×10^6) were harvested at 1, 4, and 6 hr after UVB irradiation (100 J/m^2). Total cellular RNA was extracted from 1×10^6 cells using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was performed using 1 μg of total RNA in a first-strand complementary DNA synthesis reaction with AMV Reverse Transcriptase (Promega, Madison, WI, USA). The primer used for the RT-PCR was as follows: 5′-CCCCACT GGGACACTTTCTA-3′ (forward) and 5′-TGGCCCTT TAGGTACTGTGG -3’ (reverse) for *IL-22Rα* (243 bp); 5′-CATTGGGAATGG TACCAC-3′ (forward) and 5′-CCAATAATGGTGTCATCCAC-3’ (reverse) for *IL-10Rβ* (291 bp). The PCR amplification process consisted of 35 cycles of 94°C for

30 s; 58°C for 30 s; and 72°C for 30 s for *IL-22Rα*; 35 cycles of 94°C for 30 s; 52°C for 30 s; and 72°C for 30 s for *IL-10Rβ*. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by staining with ethidium bromide.

Confocal microscopy The distribution of IL-22Rα in keratinocytes was assessed by confocal microscopy. Cells (3×10^5) were grown in 1 cm^2 coverglass at 37°C in an atmosphere of 5% CO_2 for 12 hrs. After washing three times with PBS, cells were irradiated with 100 J/m^2 of UVB and cultured for another 3 hrs and 6 hrs. Cells were then collected, fixed with 4% paraformaldehyde (PFA) and pre-incubated with 5% goat serum in PBS-T (0.3% Triton X-100 in PBS) for 1 hr. Rabbit developed anti-human IL-22Rα antibody (Ab) (abcam, cambridge, UK) was used as primary Ab and Alexa Fluor 633-conjugated anti-rabbit Ab (Invitrogen) was used as secondary Ab.

Cell proliferation assay Cells (1×10^6) were irradiated with 100 J/m^2 of UVB and then re-plated in 96-well culture plate (5×10^3 cells/well) in triplicates. At the same time, cells without UVB irradiation were also re-plated as a control. After stabilization, cells were incubated for another 24 hrs in the presence or absence of recombinant IL-22 (rIL-22) (20 ng/ml) (R&D SYSTEMS, Minneapolis, MN, USA). Ten microliters of CCK-8 solution were added to

each well of the plate, and then cell proliferation was measured by Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). The absorbance at 450 nm was normalized using the SoftmaxPro software (Molecular Devices, Sunnyvale, CA, USA).

Western blot Cells (1×10^6) were lysed and proteins were extracted using lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktails. The protein concentration was measured with ND-1000 UV/Vis (Thermo scientific, Rockford, IL, USA). An equal amount of protein (20-30 μg /sample) was dissolved in a 12% polyacrylamide-SDS gel with 100 V for 4 hrs and transferred onto a nitrocellulose membrane. Blocking was performed for 1 hr at room temperature (RT) with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST). The blocked membrane was incubated with anti-IL-22Rα Ab (1:4,000; abcam), anti-pAkt Ab (1:1,000; Cell signaling Technology, Boston, MA, USA), anti-Akt Ab (1:1,000; Cell signaling Technology) and anti-β-actin (1:8,000; Sigma) overnight at 4^{o}C. After washing 3 times (5 min/each) with PBST, membrane was incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (1:15,000; Cell signaling Technology) for IL-22Rα, pAkt, Akt and HRP-conjugated anti-mouse IgG (1:10,000; Cell signaling Technology) for β-actin for 1 hr at RT as a secondary Ab. The

membrane was then washed 3 times (5 min/each) and the immunoreactive proteins were visualized with the electrochemical luminescence (ECL) detection system (Thermo scientific). The bands were analyzed for their density using the Image J software (NIH, Bethesda, MD, USA). Results were expressed as relative intensity and each band was adjusted to that of β-actin.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) PBMCs were obtained from blood of healthy individuals with density gradient centrifugation by using Ficoll-Paque^{TM} PLUS (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The pellet was harvested and re-suspended in Red blood cell (RBC) lysis buffer (Sigma) for 5 min at 37°C. The numbers of PBMCs were counted after washing twice with PBS. To induce IL-22 production, PBMCs (2.5×10^6/ml) were stimulated with or without 5 μg/ml of Con A and cultured in complete RPMI1640 media containing 10% heat-inactivated fetal bovine serum (Life Technologies) and antibiotics (100 U/ml of penicillin and 100 μg/ml streptomycin; Life Technologies) for 48 hrs. The amounts of IL-22 in culture supernatant were measured by IL-22 ELISA kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instruction. Culture supernatant was used for the stimulation of increased IL-22Rα on UVB-irradiated HaCaT.

IL-22 bioassay Supernatant from cultured PBMCs after stimulation with or without 5 μg/ml of Con A was 10-fold concentrated through ultrafiltration using Ultracel YM-10 (Millipore, Carrigtwohill, Ireland). After filtration of concentrated supernatant with 0.22 μm pore-sized microfilter, it was treated on HaCaT with or without UVB irradiation in 96-well plate for 24 hrs. Bioactivity of IL-22 in culture supernatant to IL-22Rα on UVB-irradiated HaCaT was measured by CCK-8 based proliferation assay. To analyze the effect of IL-22 on the proliferation of UVB-irradiated HaCaT via the stimulation of IL-22Rα, 20 ng/ml of recombinant human IL-22 (R&D systems) and 2.5 μg/ml of anti-human IL-22 neutralizing Ab (R&D systems) were used.

Examination of signaling pathways for IL-22Rα expression Cells (7.5×10^5) were pre-treated with the specific inhibitors for ERK (PD98059; 20 μM; Sigma), JNK (SP600125; 20 μM; Sigma), PI3K/Akt (LY294002; 10 μM; Sigma), p38 MAPK (SB203580; 20 μM; CALBIOCHEM, San Diego, CA, USA) and NF-κB (Bay11-7082; 5 μM; Sigma) for 1 hr. After wash with PBS, cells were exposed to UVB (100 J/m^2) irradiation and cultured for another 6 hrs. Then, cells were collected and homogenized with lysis buffer. The change of *IL-22Rα* expression upon treatment of specific inhibitors in UVB-irradiated HaCaT was examined by RT-PCR and western blot as previously described.

Enzyme-Linked Immunosorbent Assay (ELISA) Cells (7.5×10^5) were exposed to UVB (100 J/m^2) and cultured for 24 hrs in the presence or absence of rIL-22 (20 ng/ml). The concentration of IL-1α, IL-6 and IL-18 in the culture supernatants was measured by ELISA (R&D systems). ELISA was performed according to the manufacturer's instruction and the relative absorbance was measured at 450 nm using the SoftmaxPro software (Molecular Devices).

Statistical analysis Values are represented as means ± SDs. Unpaired two-tailed Student's *t*-test was used to compare two groups. Statistical analysis was carried out using GraphPad InStat version 5.01 (GraphPad Software, La Jolla, CA, USA)

Results

1. UVB irradiation suppresses the growth of human keratinocyte cell line, HaCaT in a dose-dependent manner

To determine the optimal dose of UVB without cytotoxicity, I did dose kinetic study as shown in Fig. 1. After cells (7.5×10^5) were exposed to 100, 150, and 200 J/m^2 of UVB and incubated for 24 hrs, and then cell viability was examined by trypan blue dye exclusion assay. As a result, there were no remarkable differences among three doses of UVB (Fig. 1A). However, I observed the decrease of cell viability of cells that are exposed to 150 and 200 J/m^2 at 36 and 48 hr after UVB irradiation (data not shown). It suggests that the damage by 150 and 200 J/m^2 of UVB irradiation is already induced in HaCaT at 24 hr, even though it was not determined by trypan blue dye exclusion assay. Therefore, I performed our experiment with 100 J/m^2 of UVB irradiation in the present study. Next, the effect of UVB irradiation on the growth of cells was observed. As shown in Fig. 1B, proliferation was definitely suppressed by three doses of UVB, when it was compared with control. Taken together, cell proliferation was suppressed by 100, 150, and 200 J/m^2 of UVB irradiation, but there was no cytotoxic effect on cells by 100 J/m^2 of UVB irradiation only.

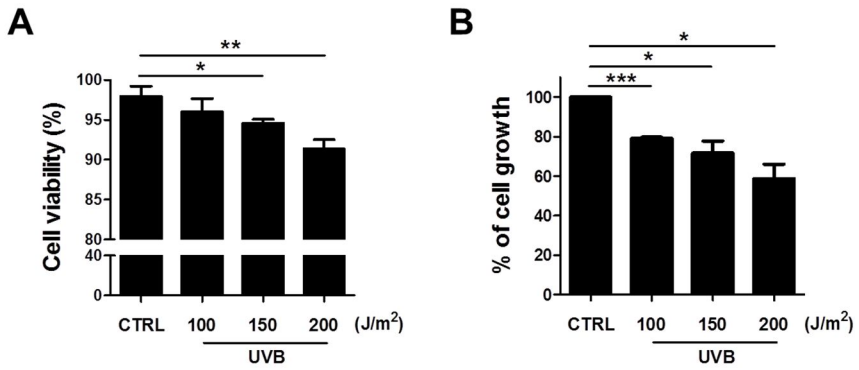

Fig. 1 The effect of UVB irradiation on the viability and proliferation of human keratinocyte cell line, HaCaT

Cells (7.5×10^5) were exposed to 100, 150, and 200 J/m^2 of UVB and then cultured for 24 hrs. The effect of UVB on the viability (A) and % growth of cells (B) were then examined by trypan blue dye exclusion assay as described in *Materials and Methods*. The formula of cell viability and % growth of cells are; cell viability (%) = (the numbers of live cells/ the numbers of total cells) × 100, % of cell growth= (the numbers of live cells at 24 hr after UVB irradiation/ the numbers of live cells at 24 hr without UVB irradiation) × 100. Results are representative of three independent experiments. Each sample is in triplicates and data are presented as the means ± SD.

2. UVB irradiation increases the expression of IL-22Rα in human keratinocyte cell line, HaCaT

It is still unclear whether IL-22Rα expression is increased during skin inflammation such as psoriasis by UVB irradiation (33). First, I examined the change on the expression of *IL-22Rα* mRNA in HaCaT after exposure to 100 J/m^2 of UVB. *IL-22Rα* mRNA expression was increased in a time-dependent manner of UVB irradiation, whereas there was no change on mRNA expression of *IL-10Rβ* (Fig. 2A). The relative expression of the both receptor mRNAs against *β-actin* are shown in Fig. 2B. Based on the results regarding the mRNA expression of *IL-22Rα* by UVB (100 J/m^2) irradiation (Fig. 2), the expression of IL-22Rα on the surface of UVB-irradiated HaCaT was examined by western blot analysis. As shown in Fig. 3, it was peaked at 24 hrs and decreased at 48 hrs after UVB irradiation. Taken together, UVB irradiation increases the expression of IL-22Rα in human keratinocyte cell line, HaCaT.

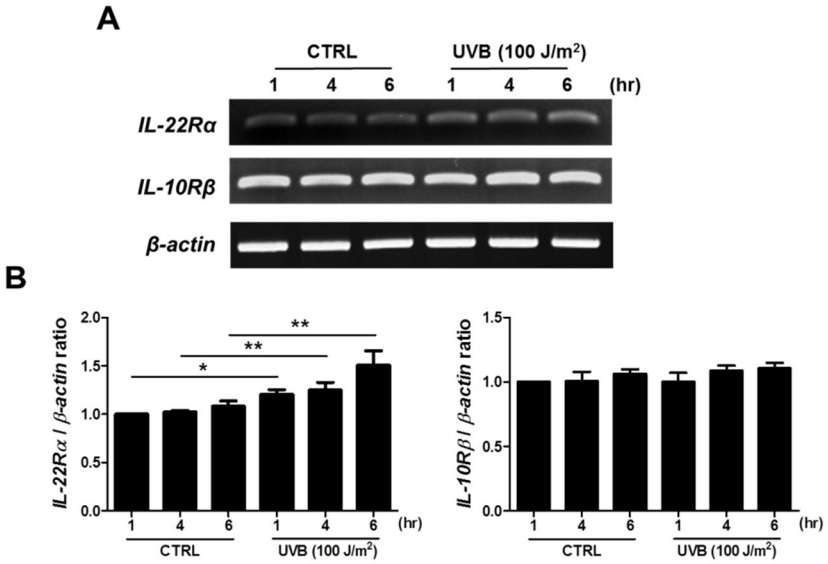

Fig. 2 The effect of UVB irradiation on the expression of *IL-22Rα* mRNA in human keratinocyte cell line, HaCaT

Cells (1×10^6) were collected at 1, 4 and 6 hrs after irradiation with 100 J/m^2 of UVB. And then, total RNA was extracted and cDNA was made. RT-PCR was performed by using the specific probe for *IL-22Rα* and *IL-10Rβ* as described in *Materials and Methods.* (A) The expression of *IL-22Rα* and *IL-10Rβ* after electrophoresis PCR product on 1.5% agarose gel and visualization by staining with ethidium bromide. (B) Densitometry analysis for the relative expression of each band against that of *β-actin*. Results are representative of three independent experiments.

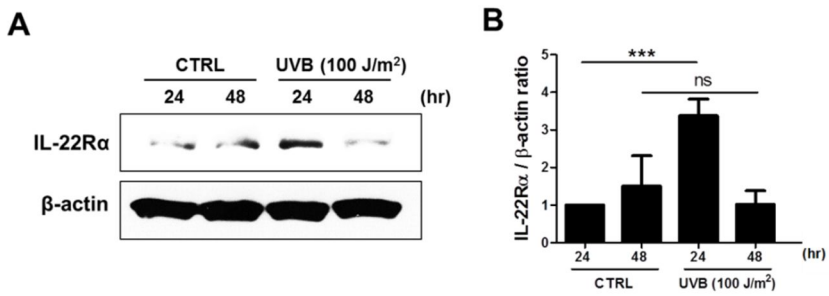

Fig. 3 The effect of UVB irradiation on IL-22Rα on the surface of human keratinocyte cell line, HaCaT

Cells (1×10^6) were collected at 24 and 48 hr after irradiation with 100 J/m^2 of UVB. Then, cells were lysed and protein was extracted for western blot analysis as described in *Materials and Methods*. (A) The expression of IL-22Rα on the surface of UVB-irradiated HaCaT. (B) Densitometry analysis for the relative expression of each band against that of β-actin. Results are representative of three independent experiments.

3. UVB irradiation activates PI3K/Akt, but it is not involved in the regulation of the IL-22Rα expression in human keratinocyte cell line, HaCaT

It is well recognized that growth factors, such as pro-survival factors, activate cell survival signaling pathway via activation of phosphoinositol 3-kinase (PI3K) and Akt (39). Therefore, I investigated whether PI3K/Akt is also involved in the expression of IL-22Rα by UVB irradiation. It was performed through pre-treatment with LY294002, a specific inhibitor for PI3K/Akt, prior to UVB irradiation. In addition, it was examined what kind of intracellular signaling molecule is involved in this process by using SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), and Bay11-7082 (NF-κB inhibitor). As shown in Fig. 4, I found that none of the inhibitors showed the regulatory effect on the expression of *IL-22Rα* by UVB irradiation. However, the phosphorylation of Akt by 100 J/m^2 of UVB was increased by UVB irradiation (Fig. 5).

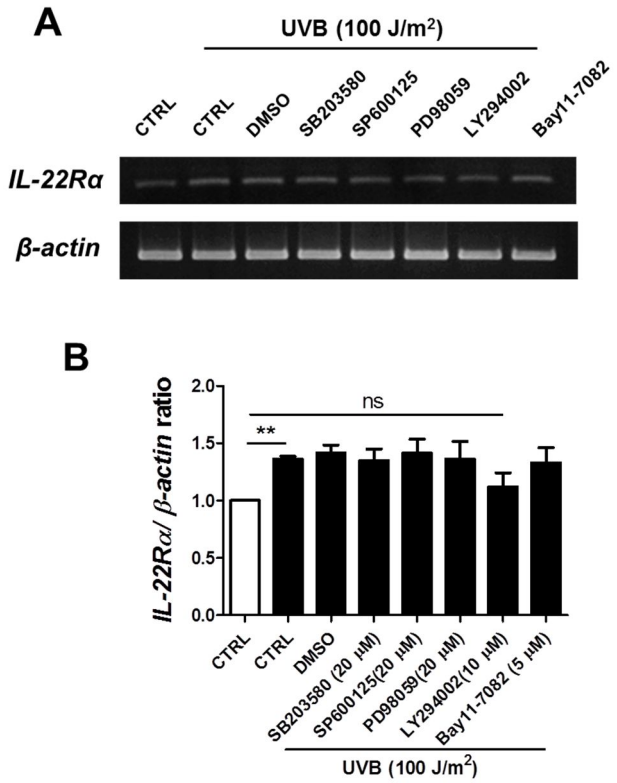

Fig. 4 PI3K/Akt independent expression of *IL-22Rα* mRNA expression in human keratinocyte cell line, HaCaT by UVB irradiation

(A) Cells (7.5×10^5) were pre-treated with DMSO (vehicle control), SB203580 (20 μM), SP600125 (20 μM), PD98059 (20 μM), LY294002 (10 μM) and Bay11-7082 (5 μM) for 1 hr and then exposed to 100 J/m^2 of UVB. After culturing for another 6 hrs, total RNA was extracted and cDNA was made. RT-PCR was performed by using the specific primer for *IL-22Rα* as described in *Materials and Methods.* (A) The expression of *IL-22Rα* after electrophoresis PCR product on 1.5% agarose gel and visualization by

staining with ethidium bromide. (B) Densitometry analysis for the relative expression of each band against that of β-actin. Results are representative of three independent experiments.

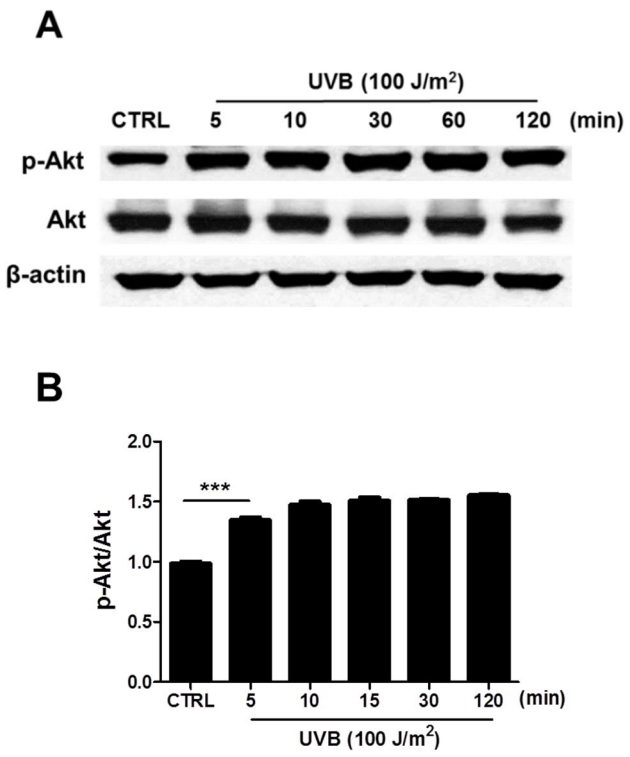

Fig. 5 Increase of the phosphorylation of Akt in human keratinocyte cell line, HaCaT by UVB irradiation

Cells (7.5×10^5) were exposed to 100 J/m^2 of UVB and then collected at indicated time points. Cells were lysed and protein was extracted for western blot analysis as described in *Materials and Methods*. (A) The change on the expression of Akt and its phosphorylation in UVB-irradiated HaCaT. (B) Densitometry analysis for the relative expression of pAkt against that of Akt. Results are representative of three independent experiments.

4. UVB induces the translocation of IL-22Rα from cytosol to membrane of human keratinocyte cell line, HaCaT

As I previously showed, *IL-22Rα* expression is increased by UVB irradiation (Fig. 2 and 3), even though it was independent on the activation of PI3K/Akt (Fig. 4). In our previous report, we found the translocation of sodium-dependent vitamin C transporter (SVCT)-1 from cytosol to membrane on human keratinocyte cell line, HaCaT by UVB irradiation (16). I examined the movement of IL-22Rα from cytosol to membrane in UVB-irradiated HaCaT by confocal microscopy. Like SVCT-1, translocation of IL-22Rα onto membrane in HaCaT was induced by UVB irradiation (Fig. 6). It suggests that the expression of IL-22Rα is also regulated by UVB irradiation at post-trascriptional level.

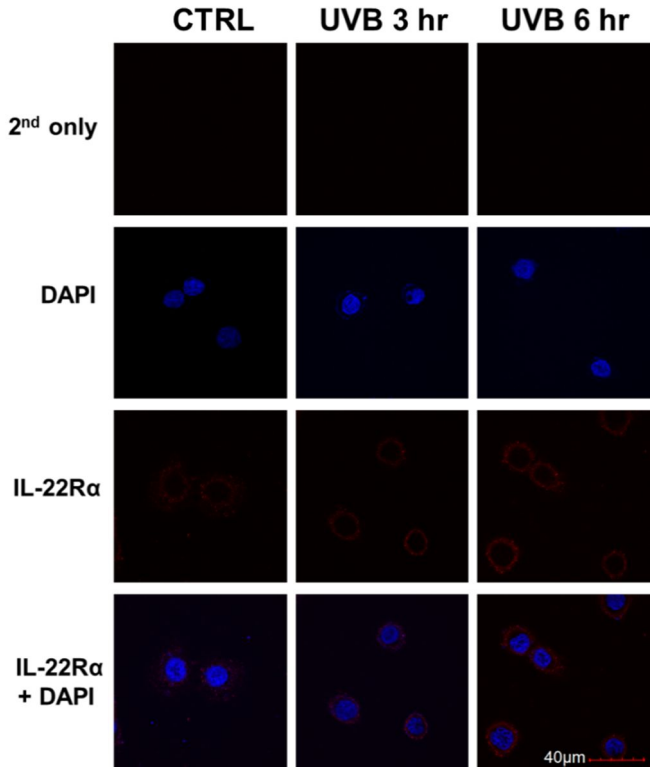

Fig.6 Post-transcriptional regulation of IL-22Rα expression in human keratinocyte cell line, HaCaT by UVB irradiation

After cells (3×10^5) were exposed to 100 J/m^2 of UVB, cells were collected at 3 and 6 hr after UVB irradiation and fixed with 4% PFA for confocal microscopy staining as described in *Materials and Methods*. Briefly, fixed cells were stained with rabbit developed anti-human IL-22Rα Ab (1:100). Alexa Fluor 633-conjugated anti-rabbit Ab was used as secondary Ab (1:1,500). Nucleus was counter-stained with DAPI (5 μl). The distribution of IL-22Rα was investigated by using confocal microscopy. Results are representative of three independent experiments.

5. IL-22 increases the suppressed proliferation of UVB-irradiated HaCaT

It is widely known that cell cycle progression can be arrested at certain checkpoints which serve to monitor the integrity of the chromosomes in response to UVB (40, 41). In addition, there is a recent report that IL-22 increases the proliferation of human keratinocyte cell line, HaCaT through IL22Rα (31). I have already shown that the proliferation of HaCaT was decreased by UVB irradiation (Fig.1), but the expression of IL-22Rα is increased at both transcriptional and post-transcriptional level in Fig. 2, 3 and 6. Therefore, I investigated whether IL-22 could recover the suppressed proliferation of HaCaT by UVB irradiation through the interaction with its increased receptor on UVB-irradiated HaCaT. When cells were cultured in the presence of rIL-22 (20 ng/ml) for 24 hrs after exposure to 100 J/m^2 of UVB, I observed that the decreased proliferation of UVB-irradiated HaCaT was recovered by rIL-22. But, there was no remarkable proliferation on control (Fig. 7).

Next, I did the experiment by using the culture supernatant of activated peripheral blood mononuclear cells (PBMCs) to confirm the effect of IL-22 on the recovery of the suppressed proliferation of UVB-irradiated HaCaT, because IL-22 is mainly produced by activated $CD4^+$ T cells, NK cells and other immune cells (20, 21). It is known that concanavalin A (Con A) stimulates the $CD4^+$ T, NK and NKT cells in a polyclonal manner, inducing

various cytokines from immune cells (42). After PBMCs were obtained from healthy individuals and activated with Con A (5 μg/ml) for 48 hrs, I confirmed the increase of IL-22 in response to Con A in culture supernatants of activated PBMCs than in those of control (Fig. 8). Culture supernatants containing IL-22 was 10-fold concentrated and then treated to HaCaT with or without UVB irradiation in the presence or absence of human IL-22Ab (2.5 μg/ml). As shown in the result by rIL-22, I found that the proliferation of UVB-irradiated HaCaT was also increased by the treatment of concentrated culture supernatant Con A-induced activated PBMCs (Fig. 9). It suggests that the proliferation of UVB-irradiated HaCaT by IL-22 might also occur in the skin. Taken together, it seems that IL-22 could increase the proliferation of UVB-damaged HaCaT.

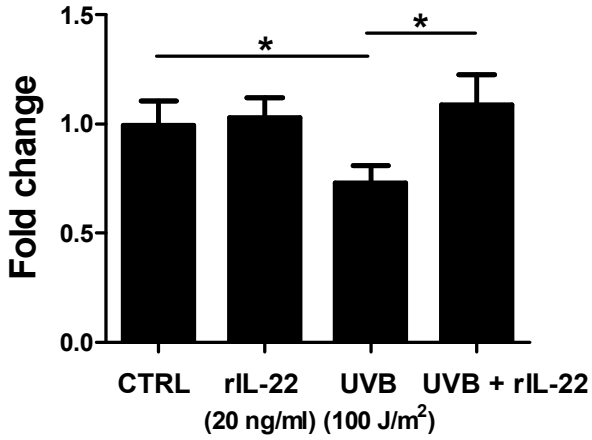

Fig. 7 Rescue of the suppressed proliferation of UVB-irradiated HaCaT by the treatment of rIL-22

After cells (1×10^6) were exposed to 100 J/m^{2}of UVB, they were cultured in the presence or absence of human rIL-22 (20 ng/ml) for 24 hrs. The change on cell proliferation was measured with Cell Counting Kit-8 (CCK-8) as described in *Materials and Methods*. The proliferation of each experimental group is normalized with the proliferation in control that is without UVB irradiation. Results are representative of five independent experiments.

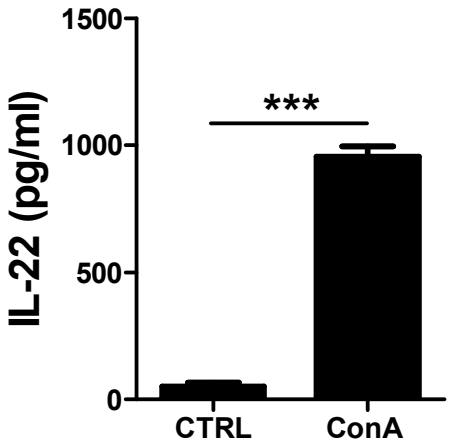

Fig. 8 The production of IL-22 in Con A-activated PBMCs

PBMCs (2.5×10^6cells/ml) obtained from healthy individuals were cultured in the presence or absence of Con A (5 μg/ml) for 48 hrs. Culture media was collected and centrifuged at 600g for 10 min. The supernatants were harvested and IL-22 amounts were determined by ELISA. Data are presents as the means ± SD.

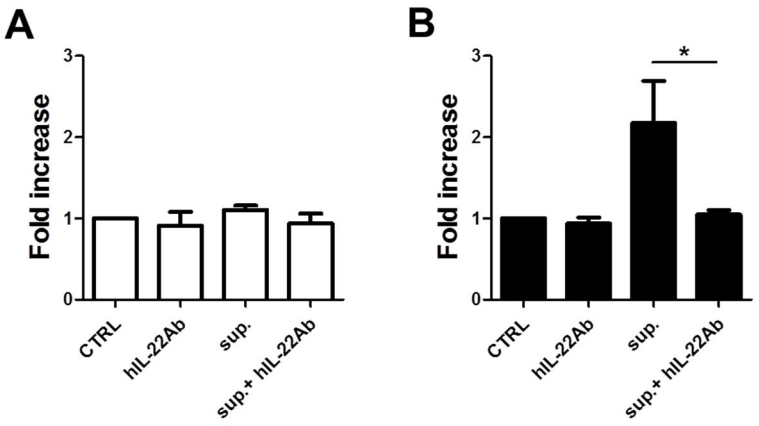

Fig. 9 The increase of the proliferation of UVB-irradiated HaCaT by the treatment of culture supernatant from activated PBMCs

The culture supernatant of activated PBMCs was 10x-concentrated and treated to cells in the presence or absence of hIL-22Ab (2.5 μg/ml) in 96-well plate. The effect of culture supernatant containing IL-22 on the change in the proliferation of cells was measured with CCK-8. The proliferation of each experimental group is normalized with the proliferation in control that is without UVB (A) or with UVB irradiation (B). Results are representative of five independent experiments. Each sample is in quadruplicates and results are representative of triplicate experiments. Data are presented as the means ± SD of fold change.

6. IL-22 increases the production of IL-1α, IL-6 and IL-18 in UVB-irradiated HaCaT

It is known that IL-22 is involved in the induction of inflammatory response (24, 26). In addition, it is well-known that the production of IL-1α, IL-6 and IL-18 is enhanced from keratinocytes by UVB irradiation (11). Moreover, those cytokines are increased in the skin after exposure to UVB (13, 16). Therefore, I investigated the role of IL-22 on the production of inflammatory cytokines from UVB-irradiated HaCaT. As I expected, the production of IL-1α, IL-6 and IL-18 in UVB-irradiated HaCaT was additionally and definitely increased at 24 hr after the treatment with rIL-22 (20 ng/ml) (Fig. 10A-C). In conclusion, IL-22 is involved in UVB-mediated inflammatory process in the skin through the increase of cell proliferation as well as the increase of the production of inflammatory cytokines.

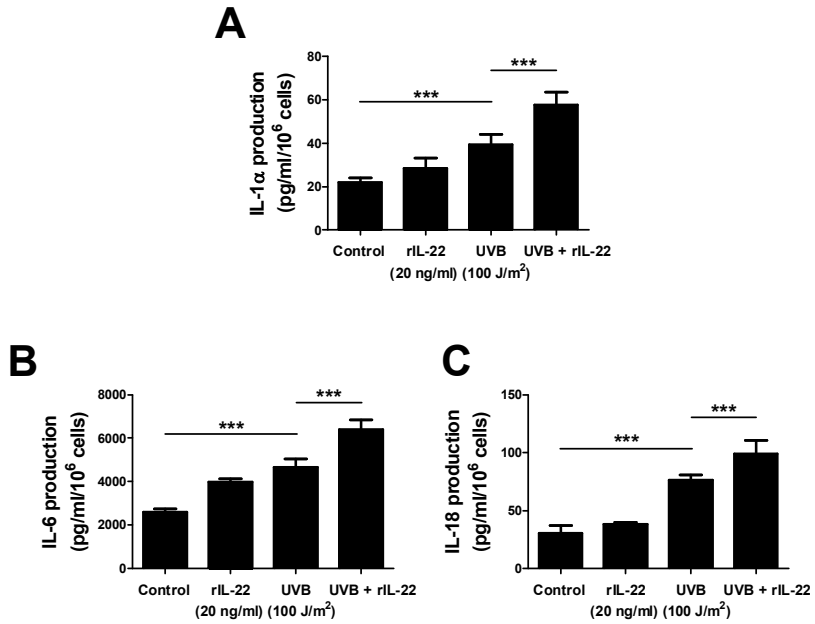

Fig. 10 IL-22 increases the production of IL-1α, IL-6 and IL-18 in UVB-irradiated HaCaT

Cells (7.5×10^5) were exposed to 100 J/m^2 of UVB and cultured for another 24 hrs in the absence or presence of rIL-22 (20 ng/ml). Culture supernatants were collected and the change in the production of IL-1α, IL-6 and IL-18 was measured by ELISA as described in the *Materials and Methods.* Each sample is in triplicates and results are representative of three independent experiments. Data are presented as the means ± SD.

DISCUSSION

Skin is considered as the largest organ in human body. Our body is covered by skin that consists of three layers: epidermis, dermis and subcutaneous layer (43). Epidermis is composed of the outermost layers of cells in the skin and is made up of keratinocytes (44). The role of keratinocytes is the secretion of various cytokines in response to stimuli such as UVB and bacterial infection (45, 46). It is regarded as an important process for cutaneous inflammatory response and homeostasis in the skin.

IL-22 is a cytokine that is produced by immune cell subsets, such as activated $CD4^+$ T cells and NK cells (20, 21). The expression of its receptor is restricted to non-hematopoietic cells in the skin, pancreas, and so on (19). There are several reports regarding the role of IL-22 on the pathogenesis of several kinds of skin diseases including psoriasis, atopic dermatitis, contact dermatitis, and scleroderma (36, 47-50). Skin disease that is the most known about the role of IL-22 on the pathogenesis is psoriasis (51-53). IL-22 expression is up-regulated in psoriatic skin lesions compared to normal skin, but its expression is obviously decreased after anti-psoriatic therapy (52). In addition, the expression of IL-22 receptor is increased in the psoriatic epidermis compared to normal epidermis (54, 55). It suggests that Th22 cell

that produces IL-22 is a major pathogenic cell in psoriasis, but it is still not known what kinds of factors are involved in the production of IL-22 from activated T cells in psoriasis. There is a recent report that an antimicrobial peptide, LL-37 modulates pro-inflammatory responses in the skin (48). That is to say, bacterial infection is one of the underlying mechanisms on the pathogenesis of skin disease via the IL-22 production. As shown in Fig. 8, IL-22 production from PBMCs is definitely increased via the stimulation with Con A. In addition, I showed increased IL-22Rα expression on UVB-irradiated HaCaT (Fig. 2-3). Considering that the repetitive exposure of skin to UVB induces sunburn and the decrease in protective potential of skin against bacterial infection, our results provide a novel mechanism of inflammatory mechanisms in the skin by UVB irradiation through the increase of IL-22 production and its receptor expression. However, direct effect of UVB on the production of IL-22 from T cells in the skin should be further investigated.

In the past few years, skin diseases related to UV rays, UVA and UVB, are gradually increasing since ozone depletion is rapidly proceeding. However, UVA is not involved in the development of skin cancer, but involved in chronic inflammation (2, 3). On the contrary, skin cancers are mostly caused by chronic exposure to UVB because it induces activation of

oncogenes and inactivation of tumor suppressor genes (6, 56). In general, it is known that cell cycle progression can be arrested at certain checkpoints which serve to monitor the integrity of the chromosomes in response to UVB (40, 41). It is considered as an effective way for preventing the proliferation of UVB-damaged cells, because the proliferation of UVB-damaged cells results in the development of skin cancer. Moreover, there is a recent report that IL-22 increases the proliferation of human keratinocyte cell line, HaCaT through IL-22R (28). The role of IL-22 and its receptor on the development and the pathogenesis of skin cancer is still largely unknown. However, I present the rescue of the suppressed proliferation of UVB-irradiated HaCaT by the treatment of rIL-22 and the culture supernatant containing IL-22 (Fig. 7 and 9). Therefore, our results suggest new insight into skin cancer development by UVB irradiation. Since I have already observed the expression of IL-22R on the melanoma (data not shown), even though IL-22Rα expression on squamous cell carcinoma (SCC), which is originated from keratinocyte, was not examined, there must be a close relationship between the proliferation or metastasis of melanoma or SCC and IL-22Rα expression on their surface. Therefore, analysis of the role of IL-22 on the progression of melanoma and SCC and the potential of IL-22Rα as a therapeutic target of melanoma is also necessary for our further experiment.

There have been reports that the translocation of sodium-dependent vitamin C transporter (SVCT)-1 from cytosol to membrane of keratinocytes by UVB irradiation (16). As a result, the uptake of vitamin C into UVB-irradiated keratinocyte was definitely increased and it results in the suppression of further inflammatory responses, such as the production of prostaglandin E2 and inflammatory cytokines. Like SVCT-1, IL-22Rα also showed increased translocation by UVB irradiation. However, it was not for the prevention of inflammation, but for the facilitation of those actions. Even though the results of the action are quite different, it seems that the translocation from cytosol to membrane is a common characteristic of the molecules that show acute response to UVB.

With regard to the expression of IL-22Rα, I previously reported that PI3K/Akt pathway is related to the expression of IL-22Rα on the hepatocytes in response to acute inflammation induced by Con A (35). In order to investigate the signaling pathway involved in IL-22Rα expression UVB-irradiated HaCaT, I performed the inhibitor study by using several kinds of inhibitors for intracellular kinases (Fig. 4). I expected the suppression of IL-22Ra expression by pre-treatment of LY294002, specific inhibitor for PI3K/Akt, but there was no remarkable change. However, I observed that phosphorylation on Akt was increased by UVB irradiation (Fig.5). Therefore,

the activation of PI3K/Akt in HaCaT is increased by UVB irradiation, but it is not related with the increased IL-22Rα expression. The role of activated PI3K/Akt in UVB-irradiated HaCaT and signaling molecule that is involved in the expression of IL-22Rα on its surface are now under investigation.

Since IL-1 and IL-6 are closely related with skin disorders caused by the proliferation of keratinocytes, regulating the production of IL-1 and IL-6 is very important (57-59). As shown in Fig. 10, the production of pro-inflammatory cytokines, IL-1α, -6, and -18, was increased from UVB-irradiated HaCaT by the treatment of IL-22. Hence, it suggests that IL-22 has direct inflammatory activity through the stimulation of IL-22Rα as well as indirect activity that is mediated by pro-inflammatory cytokines. In fact, IL-22 up-regulates the expression of CXCL8 and IL-6 in skin of the patients with atopy dermatitis and accelerates inflammation in the skin (48). However, there is no report about the expression of IL-22R not only in the skin of experimental animal model for atopy, but also in the skin of atopy patients.

In conclusion, this study is the first report regarding the increase of IL-22Rα expression in the skin, especially on human keratinocytes, by UVB irradiation. It seems that the functional consequences of increased expression of IL-22Rα by UVB irradiation might strengthen the responsiveness of keratinocytes to IL-22 stimulation. The interaction between IL-22 and

increased expression of IL-22Rα on keratinocytes by UVB irradiation plays a major role in facilitating inflammatory responses in the skin through the increase of the proliferation of UVB-irradiated keratinocytes and inflammatory cytokine production. Therefore, our study provides the new insight into UVB-induced skin inflammation and regulation of related inflammatory skin diseases.

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국 문 초 록

자외선B가 조사된 사람 각질 세포주에 있어서 interleukin-22의 효과

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Interleukin (IL)-22는 IL-10 가계에 속하며, 염증 반응의 잠재적인 매개자이다. 이것은 주로 활성화된 $CD4^+$ T세포와 자연살해세포에서 분비되며, IL-22 수용체α는 피부, 췌장, 소장, 간, 폐와 신장 등의 비조혈모세포에서 발현되어 있다. 최근의 보고에 따르면, IL-22가 각질세포의 세포주기를 조절함으로써 상피의 항상성유지에 중요한 역할을 한다고 밝혀진 바가 있다. 게다가 자외선B는 피부 염증을 일으킨다고 이미 알려져 있다. 그러나, 자외선 B와 IL-22의 생산 및 그 수용체 발현에 관해서는 알려진 바가 없다. 그러므로, 우리는 자외선B가 조사된 사람 각질세포주인 HaCaT의 증식과 피부 염증반응 유발에 있어서 IL-22수용체α에 관한 IL-22의 역할을 규명하였다. 100 J/m^2의

자외선B를 HaCaT에 조사했을 때, IL-22 수용체α가 증가됨을 mRNA와 단백질 수준에서 확인하였다. 흥미롭게도, 자외선B 조사에 의해 세포질에서 세포막으로의 IL-22 수용체α의 이동을 확인할 수 있었다. 일반적으로 자외선B는 HaCaT의 증식을 억제한다고 알려져 있으나, 자외선B에 의해 감소된 HaCaT의 증식이 재조합 IL-22와 활성화된 말초혈액단핵세포의 배양액에 의해 회복되었다.

마지막으로, 자외선B가 조사된 HaCaT에서 염증성 사이토카인인 IL-1α, IL-6와 IL-18의 분비가 재조합IL-22처리에 의해 더욱 증가됨을 확인하였다. 따라서, IL-22는 피부 염증을 증가시키고 자외선B에 의해 증가된 IL-22 수용체α를 통하여 HaCaT의 증식을 촉진시킨다는 것을 알 수 있다.

주요어: 염증, 증식, HaCaT, IL-22, 자외선B

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